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# Copy-Number Variation and False Positive Prenatal Aneuploidy Screening Results

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## SUMMARY

Investigations of noninvasive prenatal screening for aneuploidy by analysis of circulating cell-free DNA (cfDNA) have shown high sensitivity and specificity in both high-risk and low-risk cohorts. However, the overall low incidence of aneuploidy limits the positive predictive value of these tests. Currently, the causes of false positive results are poorly understood. We investigated four pregnancies with discordant prenatal test results and found in two cases that maternal duplications on chromosome 18 were the likely cause of the discordant results. Modeling based on population-level copy-number variation supports the possibility that some false positive results of noninvasive prenatal screening may be attributable to large maternal copy-number variants. (Funded by the National Institutes of Health and others.)

Methods of noninvasive prenatal screening[1] have advanced rapidly in clinical practice, with aneuploidy screening based on analysis of circulating cfDNA now routinely offered to women with high-risk pregnancies. Owing to the high reported accuracy of these screening tests,[2,3] attention has shifted to low-risk cohorts, in which the reduced incidence of aneuploidy may limit the positive predictive value of noninvasive prenatal screening.[4] A recent prospective analysis of cfDNA-based noninvasive prenatal screening in 1914 low-risk pregnancies showed false positive rates of 0.3%, 0.2%, and 0.1% for trisomies 21, 18, and 13, respectively — rates that were lower than those observed with standard screening tests.[5] However, the positive predictive value was 45.5% for trisomy 21 and 40.0% for trisomy 18,[5] highlighting the need for follow-up diagnostic testing. Norton et al.[6] now report in the *Journal* higher positive predictive values with cfDNA-based noninvasive prenatal screening that uses a different method, albeit with a higher “no call” rate; “no call” results are ambiguous and could mask a clinically important finding.



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The mechanisms underlying false positive results of cfDNA-based noninvasive prenatal screening remain incompletely elucidated.[7] Explanatory hypotheses include maternal mosaicism,[8,9] undetected tumors,[10] the vanishing twin syndrome,[11] and confined placental mosaicism,[12,13] as well as technical errors. Although case reports have documented examples of underlying causes of false positive and other aberrant results, only a small proportion have been comprehensively explained.[8]

Methods of cfDNA-based noninvasive prenatal screening include massively multiplex polymerase-chain-reaction (PCR) assay,[14] shotgun sequencing,[15,16] and targeted sequencing.[17] The Illumina Verifi and Sequenom MaterniT21 PLUS tests are based on counting statistics that naturally arise from shotgun sequencing of total cfDNA in maternal plasma. After isolation, sequencing, and alignment of cfDNA fragments, a minority of which are fetoplacentally derived (mean, 13%, but with considerable variation during pregnancy and between pregnancies[18]), the reads are sorted into bins. Each bin contains reads that have been unambiguously derived from a specific chromosome, and the distributions for each chromosome are converted to standard normal distributions. The binned counts for the newly analyzed cfDNA sample are compared with reference distributions, yielding per-chromosome z scores that estimate the likelihood of fetal aneuploidies. In diploid pregnancies, false positive detection of trisomy may occur owing to type I errors — that is, the infrequent and chance sampling of z scores above 4.0. In statistical terms, the probability that the random variable $Z$ will have a value greater than 4.0 is expressed as $\Pr(Z>4.0)$, which equals approximately 3 in 100,000.

This approach implicitly assumes that every woman carries the same proportion of genetic material on a given chromosome. In fact, chromosomes vary slightly in composition and size from person to person owing to inherited or de novo copy-number variants, in which a genomic region is deleted or duplicated. For example, a maternal duplication effectively increases the length of the chromosome on which it resides, thereby increasing the proportion of cfDNA derived from that chromosome. In such a person, sequencing of cfDNA would yield overrepresentation of reads derived from the chromosome containing the copy-number variant relative to that chromosome in reference persons, potentially leading to false interpretation of the results as indicating fetal trisomy (Fig. 1A).

The capacity of a maternal copy-number variant to alter the interpretation of noninvasive prenatal screening is augmented by the fact that the vast majority of cfDNA is maternally derived. In a diploid pregnancy in which the mother carries a duplication, the increased number of reads derived from the additional copy of the duplicated region shifts the sampling distribution for this pregnancy to the right relative to the underlying reference distribution (Fig. 1B). The probability of a false positive statistical test would then exceed $\Pr(Z>4.0)$, with the extent of excess driven primarily by the size of the duplication.

We sought to investigate whether maternal copy-number variants could give rise to false positive results of noninvasive prenatal screening. As a proof of principle, we enrolled four pregnant women who had discordant findings: positive cfDNA-based screening results with normal clinical outcomes. Subsequently, we modeled the potential population-level effects of maternal copy-number variants on false positive results.

# METHODS

## PATIENTS AND SAMPLE PROCESSING

Participants were identified from a population of consecutive patients with false positive results on noninvasive prenatal screening who were referred for perinatal genetic counseling at the University of Washington. After delivery, normal clinical outcomes were confirmed; all the participants had fetal diploidy on the basis of antenatal genetic amniocentesis, normal newborn examinations, or both.

For each pregnancy, maternal peripheral-blood samples were obtained at the time of enrollment, and cord-blood samples were obtained at delivery. Plasma was purified, and cfDNA was isolated, sequenced, and aligned to the reference genome with the use of standard methods (see the Methods section in the Supplementary Appendix, available with the full text of this article at NEJM.org). Maternal peripheral-blood mononuclear cells (PBMCs) were obtained concurrently, and DNA was isolated from these cells for validation of copy-number variants.

## IDENTIFICATION OF COPY-NUMBER VARIANTS

Reads from maternal cfDNA unambiguously derived from each chromosome were tallied and examined for overrepresentation of portions of chromosome 13, 18, or 21. Candidate copy-number variants greater than 250 kb in size were identified by visual inspection of read-depth profiles and confirmed by means of PCR assay and Sanger sequencing of maternal PBMC DNA and cord-blood DNA (see the Methods section in the Supplementary Appendix).

## MODELING

For a range of sizes of maternal copy-number variants, we calculated the factor increase in the probability of a false positive statistical test for each chromosome, on the basis of the properties of the Z distribution underlying the test (see the Methods section in the Supplementary Appendix). Per-chromosome coefficients of variation were used to estimate the standard deviation of the number of reads derived from each chromosome in presumed diploid reference cohorts (Table S1 in the Supplementary Appendix). The mean numbers of additional reads expected to be derived from the duplicated regions were converted to chromosome-specific standard-deviation units, which were then used to calculate adjusted probabilities of false positive results. Next, we estimated the population frequencies of nonpathogenic copy-number variants on chromosomes 13, 18, and 21. To obtain this estimate, we evaluated a reference panel of copy-number variants of 19,584 persons, predominantly of European descent (Table S2 in the Supplementary Appendix), to determine duplications on relevant chromosomes with at least 50% overlap with unique genomic regions.

# RESULTS

## PATIENTS

We enrolled four participants, each with discordant results of noninvasive prenatal screening and clinical findings. In each case, noninvasive prenatal screening was performed by means of Illumina Verifi. Three participants had a positive screening result for trisomy 18, and the other participant had a positive screening result for trisomy 13 (complete clinical information is provided in Table S3 in the Supplementary Appendix). In two of the three participants with a positive screening result for trisomy 18, we identified maternal copy-number variants on chromosome 18 (Fig. 1C).

Patient 1 was a 36-year-old primigravida. Noninvasive prenatal screening at 18 weeks of gestation indicated fetal trisomy 18. Ultrasonographic findings at 20 weeks of gestation were consistent with normal fetal anatomy and concordant biometry. Diagnostic testing by means of genetic amniocentesis was consistent with a diploid male pregnancy. The remainder of the pregnancy was uncomplicated, and a healthy male infant was delivered at term.

Patient 3 was a 34-year-old multigravida. The results of noninvasive prenatal screening at 12 weeks of gestation were consistent with fetal trisomy 18. At 12, 16, and 20 weeks of gestation, fetal ultrasonography showed normal anatomy and concordant biometry. Genetic amniocentesis was declined. The remainder of the pregnancy was uncomplicated, and a healthy female infant was delivered at term.

## ANALYSIS OF CFDNA

Analysis of cfDNA from Patient 1 identified a duplicated region on chromosome 18 containing portions of 18p11.31 and 18p11.23 (1.15 Mb). Analysis of cfDNA from Patient 3 identified a duplication on chromosome 18 covering a region of 18p11.31 (487 kb) (Fig. 1C). For both patients, maternal DNA from PBMCs was used to validate the copy-number variant by means of PCR assay and Sanger sequencing (Table S4 and Fig. S1, S2, and S3 in the Supplementary Appendix).

## MODELING

To model the effect of these duplications on the risk of false positive results of noninvasive prenatal screening, we calculated the theoretical factor increase in the probability of false positive results for a range of sizes of copy-number variants on chromosomes 13, 18, and 21 (Fig. 2, and Fig. S4 in the Supplementary Appendix). The calculated increase depends on several factors, including the total number of reads per sample, the coefficient of variation for the chromosome in question,[19] the fetal fraction, and fetal inheritance of the maternal copy-number variant (Table S5 in the Supplementary Appendix). As the fetal fraction increases, the signal of overrepresentation is dampened if the copy-number variant is not transmitted to the fetus, and increasingly large duplications are necessary to reach the same factor increase in the probability of a false positive result. Conversely, if the copy-number variant is transmitted to the fetus, the maternally inherited chromosome also contributes to the signal of overrepresentation, obviating dependency on the fetal fraction. We estimate that the copy-number variant that was present in Patient 1, duplicating 1.15 Mb and

inherited by the fetus, increased the probability of a false positive statistical test on chromosome 18 by a factor of approximately 15,650, such that in the absence of fetal aneuploidy, the test was nearly equivalent to flipping a coin. The 487-kb copy-number variant that was present in Patient 3 but not inherited by the fetus had a more modest estimated effect, yielding an increase in the probability of false positive results by a factor of 128 to 262 for plausible fetal fractions of 5 to 20%.

### FACTORS CONTRIBUTING TO EFFECTS OF MATERNAL COPY-NUMBER VARIANTS

We identified two population factors that contribute to the effects of maternal copy-number variants. First, the distribution of copy-number-variant sizes varies according to chromosome length, with chromosomes 13 and 18 having higher population frequencies of large duplications than the smaller chromosome 21 (Fig. 2). Chromosomes with higher population burdens of copy-number variants — particularly the largest such variants — should be more susceptible to false positive results. Second, the coefficient of variation of sequence reads for each chromosome modulates the effect of the size of copy-number variants on the probability of false positive results. For example, chromosome 13, which has the highest of the three examined coefficients of variation, is the most buffered from the effects of copy-number variants (Fig. 2).

## DISCUSSION

Recent advances in cfDNA-based noninvasive prenatal screening have yielded screening techniques with substantially better test-performance characteristics than previous approaches.[5,6] However, the positive predictive value remains limited in both high-risk and low-risk populations, and improvement of these screening tests, including delineation of potential mechanisms of false positive results, will be essential as uptake of this form of screening continues to accelerate. We identified and validated large maternal copy-number variants on chromosome 18 as plausible causes of discordant results in two of four pregnancies with false positive results of noninvasive prenatal screening. Furthermore, using frequencies of copy-number variants from a largely European cohort, we estimated that maternal copy-number variants may contribute substantially to an elevated risk of false positive results.

Our study has several limitations. First, the study samples were not obtained at the same time as the initial samples that were sent for commercial testing; therefore, it is possible that underlying biologic changes occurring during gestation were masked. Although the presence of maternal copy-number variants is unaffected by the timing of sample collection, the effect of these copy-number variants on statistical inference of fetal ploidy does depend on fetal inheritance and the fetal fraction, the latter of which increases with gestational age. Thus, later in gestation, marginally larger copy-number variants are generally required to achieve the same factor increase in the probability of a false positive result when the copy-number variant is not transmitted (Fig. 2, and Table S5 in the Supplementary Appendix). Second, we did not directly observe any large copy-number variants underlying false positive results of noninvasive prenatal screening on chromosome 13 or 21. Third, our preliminary estimates of the effect of maternal copy-number variants from modeling based on population-wide

frequencies are only as good as the assumptions and data that went into them, which include the methods themselves (which are not necessarily the best possible or static), the coefficients of variation for each chromosome, the set of unique genomic regions that potentially harbor copy-number variants (Fig. S4 in the Supplementary Appendix), and the joint distribution of sizes of copy-number variants and allele frequencies. For example, the spectrums of sizes and frequencies of copy-number variants may differ between European and non-European populations, underscoring the importance of future studies with diverse patient groups.

A small cohort such as ours is insufficient to determine the precise effects of maternal copy-number variants on aggregate false positive rates of cfDNA-based noninvasive prenatal screening. Other cfDNA-based screening methods, such as the targeted analysis of cfDNA from selected genomic regions,[6] may be more or less susceptible to false positive results attributable to maternal copy-number variants. However, even as larger studies are warranted, implementations of noninvasive prenatal screening based on counting statistics arising from shotgun sequencing may be immediately modifiable to reduce the number of false positive results attributable to maternal copy-number variants. For example, when a maternal copy-number variant is identified (in cfDNA or PBMC-derived DNA, with the latter unconfounded by the fetus), reads derived from the affected region could be discarded or proportionally discounted, or the effective size of the chromosome could be adjusted. Alternatively, z scores could be calculated in fixed genome bin sizes, rather than for whole chromosomes, such that region-specific outliers that potentially correspond to maternal copy-number variants could be flagged or discarded; this approach is analogous to methods developed by Srinivasan et al.[20]

Our study has several potential implications for the spectrum of causes of discordant prenatal test results. First, although the incidence of fetal aneuploidy increases with maternal age, the risk of a false positive result of noninvasive prenatal screening that is caused by a maternal copy-number variant would not depend on maternal age, with affected women at risk for recurrent false positive results in subsequent pregnancies. Second, the presence of maternal copy-number losses or deletions of sequence could potentially induce the opposite effect — that is, false negative results of noninvasive prenatal screening in truly aneuploid pregnancies, although this issue has not been directly addressed in this study (Fig. 1A). Although the effect of the size of copy-number variants on hypothetical false negative results cannot be quantified without coefficients of variation based on truly aneuploid pregnancies, the co-occurrence of trisomic pregnancy and statistically relevant deletions is expected to be very infrequent.

In conclusion, although prospective studies have shown excellent performance of cfDNA-based noninvasive prenatal screening, the positive predictive value remains limited, and follow-up diagnostic testing remains essential. The effects of false positive screening results go beyond the clinical risks and financial costs of diagnostic testing and include potentially substantial psychological stress for patients. Our modeling, which is based on population-wide frequencies of copy-number variants, provides initial estimates on which larger, more definitive studies can be based. Though cfDNA-based noninvasive prenatal screening is currently focused clinically on high-risk populations,[21] it will probably be increasingly used

as a primary screening test over time. Throughout this transition, continued investigation and refinement of methodologic approaches to improve the performance of noninvasive prenatal screening will be critical.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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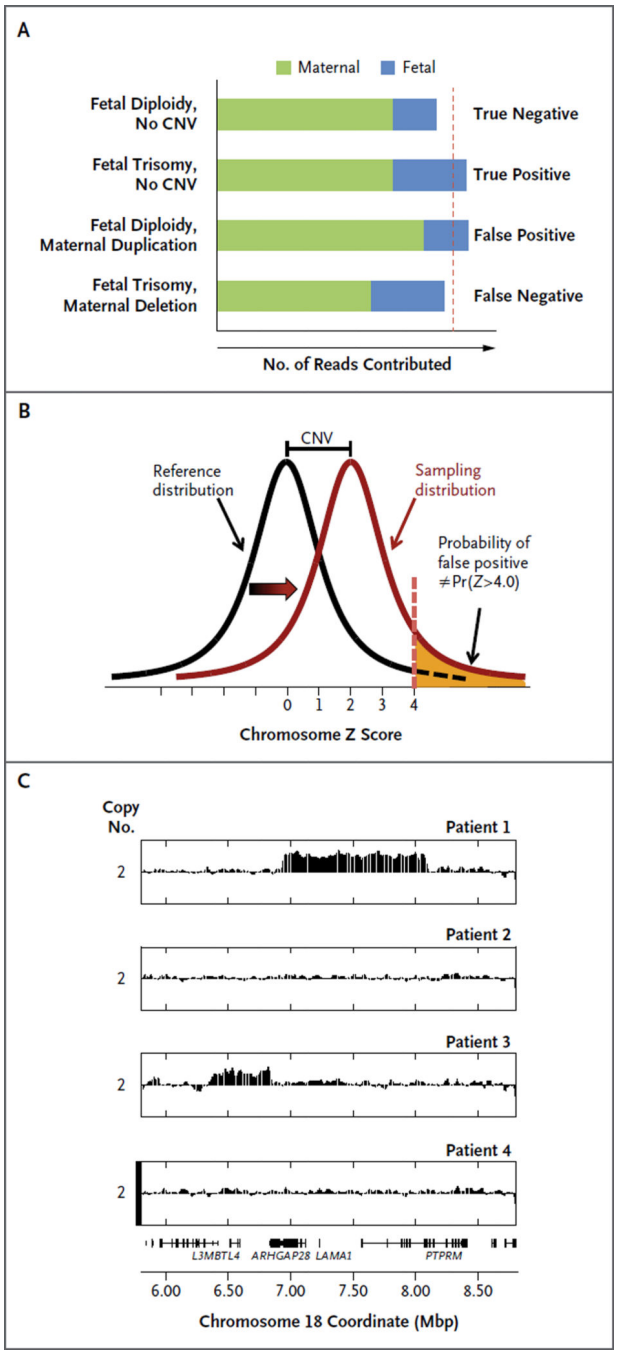


**Figure 1. The Role of Maternal Copy-Number Variants (CNVs) in False Positive Results of DNA-based Noninvasive Prenatal Screening**

Panel A is a schematic representation of cell-free DNA (cfDNA) analysis. The cfDNA in maternal plasma contains primarily maternal cfDNA and a smaller proportion of fetal cfDNA. The threshold for triggering a positive cfDNA test is indicated by the vertical dashed line. The combination of fetal diploidy and the absence of a maternal CNV results in a true negative test. The combination of fetal trisomy and the absence of a maternal CNV results in a true positive test. The combination of fetal diploidy and the presence of a maternal CNV that duplicates a portion of a relevant chromosome results in a false positive

test. Hypothetically, the combination of fetal trisomy on a specific chromosome and the presence of a maternal CNV that deletes a portion of the same chromosome could result in a false negative test. Panel B is a schematic representation of the effect of a maternal CNV on the probability of a false positive test result, expressed as Pr($Z$>4.0), which equals approximately 3 in 100,000. Maternal duplications shift the sampling distribution of the test to the right, and the underlying reference distribution is unchanged. In Panel C, copy-number profiles based on normalized cfDNA read depth are consistent with duplicated regions on chromosome 18 in two of the four patients. Profiles of Patients 2 and 4 are consistent with two copies throughout the region of interest. Patients 1 and 3 have an increased copy number in contiguous regions, suggestive of duplications.

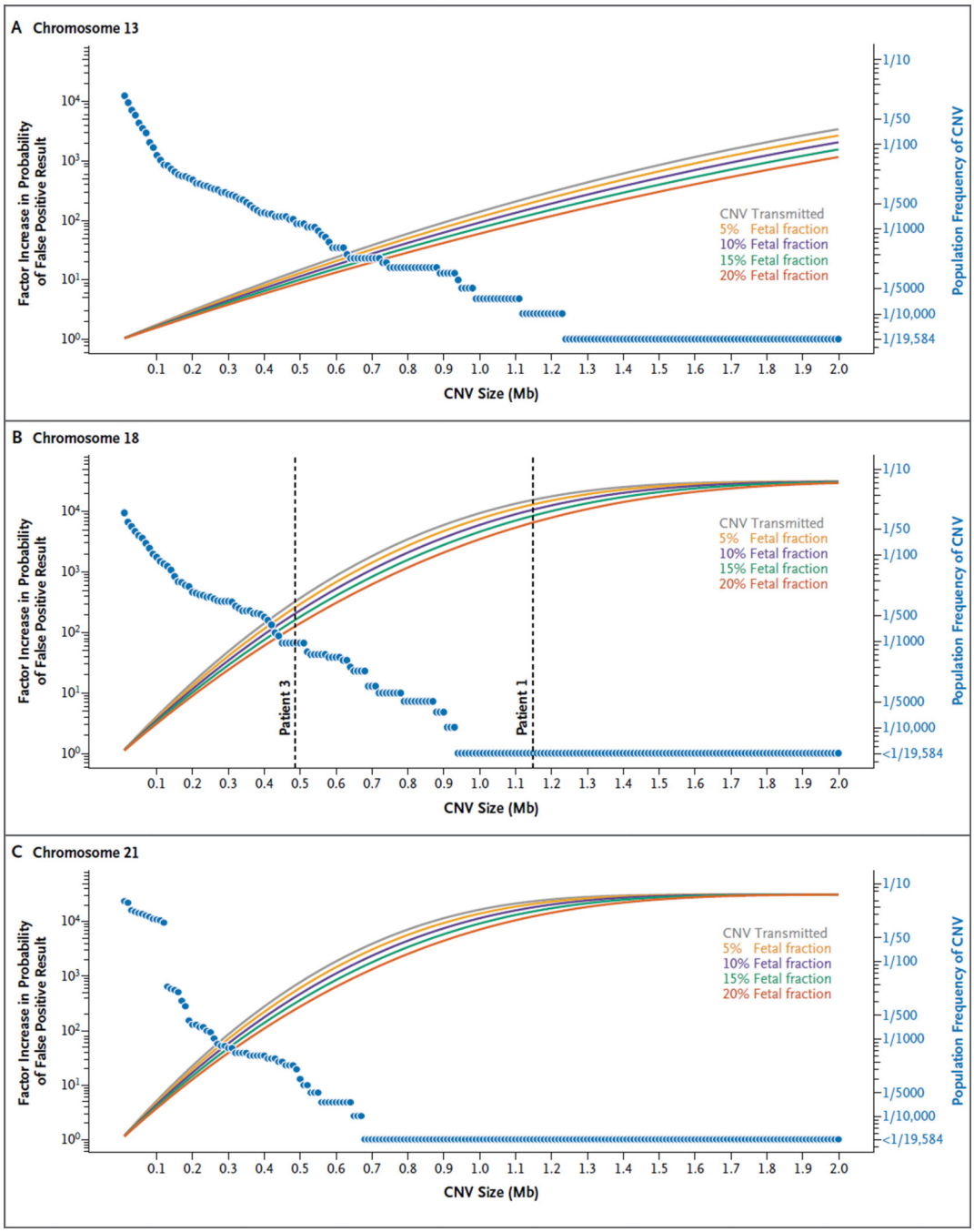


**Figure 2. Population Frequency and Estimated Effect of Maternal CNVs on False Positive Test Rates**

The burden of nonpathogenic copy-number increases on chromosomes 13 (Panel A), 18 (Panel B), and 21 (Panel C) in a cohort of 19,584 persons, predominantly of European ancestry, is shown for a range of CNV sizes (blue circles, right vertical axis). CNV frequencies in each size bin refer to CNVs of the given size or larger. For each size bin, the estimated factor increase in the probability of a false positive test resulting from the copy-number increase is shown for a range of fetal fractions (gray and colored lines, left vertical

axis). The sizes of the CNVs present on chromosome 18 in Patients 1 and 3 are highlighted (dashed vertical lines).